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Does sustained tolerance of reduced salinity seawater alter phagocytosis efficiency in haemocytes of the blue mussel *Mytilus edulis* (L.)?

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ABSTRACT

Climate change driven increases in precipitation and extreme weather events bring with them periodic introductions of large volumes of freshwater to coastal waters and the possibility of extended salinity reductions capable of challenging marine organisms living there. *Mytilus edulis* is an osmoconformer, with no physiological capacity to maintain its haemolymph osmolarity above that of a dilute seawater medium. These mussels can however tolerate reduced seawater salinity to some degree by using intracellular volume control mechanisms to extend the range of seawater salinity they can tolerate, allowing them to continue to feed, respire and maintain general cellular function in fluctuating salinity conditions. When *M. edulis* are exposed to a falling salinity gradient, a critical salinity is reached that triggers closure of the valves, isolating their internal tissues from the surrounding environment and protecting them from further osmotic stress. This study investigated whether sustained tolerance of *M. edulis* to seawater with salinity just higher than the threshold level for valve closure affected haemocyte phagocytosis efficiency. Phagocytosis by haemocytes plays a significant role in the immune system of *M. edulis* by removing foreign bodies such as invading micro-organisms, together with dead and dying cells from the haemolymph. Valve gaping behaviour was recorded continuously in mussels exposed to a reducing seawater salinity gradient to establish the critical level initiating valve closure. *M. edulis* were then held for 5 d within a flow through seawater system at a salinity of 25, close to the trigger value for valve closure in the population of mussels investigated (Namsenfjord, Norway). The exposed mussels continued to hold their valves open and active throughout, though mean valve gape and movement between valves were generally lower than control mussels held at ambient seawater with salinity of 34.3. Post-exposure total haemocyte counts and haemolymph protein concentrations in exposed mussels were similar to the controls, while phagocytosis performance, measured using flow cytometry, improved in the reduced salinity exposed mussels. These findings support a conclusion that *M. edulis* have the physiological capacity to continue to irrigate their gills for at least 5 days when held continuously at seawater salinity close to their valve closure trigger level without detriment to haemocyte phagocytosis efficiency, a key component of their immune-response system. Such a capability will be important in the event of increasing persistent reduced salinity episodes.

1. Introduction

Increases in storm frequency and precipitation levels associated with climate change have been recorded in some geographical regions, including the coasts of Norway (OECD, 2013), and are expected to increase further in coming years (Donat et al., 2016; Lehmann et al., 2015; IPCC, 2014; Philippart et al., 2011). Consequently, riverine input and runoff from land will increase the number, duration and magnitude of persistent salinity reductions in coastal waters that may affect the marine organisms that inhabit them. The blue mussel (*Mytilus edulis*) is widely distributed throughout northern European coastal waters and beyond (Tyler-Walters, 2008) and is both economically and ecologically important. It is an osmoconformer, with no physiological capacity to control extra-cellular fluid osmolarity when exposed to falling seawater salinity (Davenport, 1985; Gosling, 2003). It is however, commonly found in coastal marine habitats influenced by freshwater input where it tolerates some degree of reduced salinity by controlling cell volume with adjustment to intracellular concentrations of free amino acids and other small organic molecules (Hauton, 2016; Gosling, 2003). With progressive reduction in salinity a point is reached where cell volume adjustment is no longer able to adequately address osmotic imbalance and the valves are closed, effectively isolating the soft tissues of the mussel from the external medium (Davenport, 1979). *M. edulis* is a filter feeder and relies on water flow across its gills to deliver food and to act as conduit for respiratory gas exchange and excretion. Cessation of these processes following complete valve closure therefore has a

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direct effect on growth and fitness of individuals and creates additional energetic costs (Hauton, 2016; Wang and Widdows, 1993; Westerbom et al., 2002). Tolerating reduced salinity conditions in order to maintain water flow across gills also has energetic costs for *M. edulis* (Davenport, 1985; Maar et al., 2015; Hawkins and Hilbish, 1992), and furthermore has potential to impair the performance of cellular functions, including immune responses (Bussell et al., 2008). However, when costs and benefits are weighed against one another it is reasonable to assume that the physiological imperative for *M. edulis* will be to avoid or minimize periods of total valve closure. Short periods of salinity reduction may be managed effectively in *M. edulis* by achieving a balance between tolerance and valve closure, but future climate scenarios present the possibility of extended periods of reduced salinity.

This present study investigated valve gaping behaviour and haemocyte phagocytosis efficiency in *M. edulis* in order to determine whether tolerance to persistent low salinity conditions compromised this important aspect of their immune response. Possible major confounding effects, arising from anoxic conditions within mussels following extended periods of valve closure, were avoided by setting the test exposure salinity to fall within the tolerance window of the population of mussels used. The first objective of the study was to determine the degree of salinity reduction necessary to trigger valve closure. Once this was established, valve gape activity was monitored continuously in *M. edulis* exposed to a salinity just above this critical level for 5 days. This approach set out to establish if tolerance to reduced salinity by *M. edulis*, indicated by open and active valves, would be sustained throughout an extended period of reduced salinity, such as may follow a severe storm event. The final objective of the study was to determine if these same exposure conditions would affect phagocytosis performance. Phagocytosis by haemocytes plays a significant role in the immune system of *M. edulis* (Galloway and Depledge, 2001; Bayne, 1990; Wootton et al., 2003) by removing foreign bodies such as invading micro-organisms, together with dead and dying cells from the haemolymph. The capacity for haemocytes to perform this task was measured in *M. edulis* haemolymph using flow cytometry methodology following a 5 d exposure to the selected salinity. Additional supporting haemolymph parameters, including haemolymph protein concentration, total haemocyte count and haemolymph osmolality were also measured in both exposed and control mussels.

In summary, this study investigated how exposure to a persistent reduction in seawater salinity, at a level just above the critical valve closure threshold for the population of *M. edulis* tested, would affect ongoing valve activity and phagocytosis efficiency in this ecologically and economically important marine bivalve.

2. Materials and methods

2.1. Mytilus edulis collection and maintenance

M. edulis were purchased from a farm located in Namsenfjord in Nord-Trøndelag, Norway, in November 2015. 17 kg of adult mussels were collected and packed in a Styrofoam fish box without processing and stored at 4 °C prior to air transport to Stavanger. They arrived at the laboratory within 72 h of harvesting and were transferred to a large holding tank (420 L) fed with a continuous flow of filtered seawater (3 L min^{-1}) pumped from the fjord adjacent to the laboratory (Byfjord, Randaberg). Mussels were fed with concentrated algae solution (Instant Algae, 1800 diet) every 2–3 days. Water temperature in the tank was recorded at 10.3 ± 0.3 °C and salinity at 34 ± 0.5 throughout the holding period prior to the experimental trials.

2.2. Reduced salinity exposure system

A sequence of water vessels set within a support frame used gravity to provide a continuous regulated flow of seawater to two treatment tanks (Fig. 1). The salinity of water flowing into the first treatment tank was manipulated by the addition of freshwater that had previously passed through an activated charcoal filter to remove the possible presence of chlorine arising from prior mains water treatment. The volume of freshwater added to the mixing tank was controlled by its rate of flow through a series of silicon rubber tubes showing a stepwise increase in internal diameter (3, 4, 5, 6, 7, 8 and 9 mm). Each tube was fitted with a clamp that permitted conditions of either full or zero flow. To encourage rapid mixing, the distill ends of all freshwater tubes, together with a tube from the header tank supplying filtered seawater, were fitted within drilled holes spaced around the circumference of a plastic ring (diameter 66 mm, wall thickness 6 mm), with the open ends of all tubes directed towards its center. The completed ring assembly was positioned on the base of the mixing tank. The second treatment tank was supplied with filtered seawater only and acted as a control. The rate of water flow from the mixer tanks to the treatment tanks was 2.35 L min^{-1}, set by a combination of head height of water in the mixer tank and the internal diameter of the supply tube. Water temperature and salinity were continuously logged in each treatment tank at 1 min intervals throughout each experimental trial (DST CTD, Star-Oddi, Gardabær, Iceland). The salinity and temperature of seawater pumped in from the fjord was recorded every 5 min throughout the duration of the experiments using a CT-probe (Aqua TROLL 100®, In-Situ Inc., Collins, USA) with Win-Situ 5 data acquisition software (In-Situ Inc., Collins, USA).

2.3. Measurement of valve gape in Mytilus edulis

The distance between valves of individual *M. edulis* was continuously recorded using Hall effect proximity sensors. These sensors respond to magnetic field strength, with their output voltage changing in relation to distance from a magnetic source. A small circular neodymium magnet (5 mm diameter and 2 mm deep) provided the magnetic field and was attached with cyanoacrylate glue to the top edge of one valve, positioned midway along the length of the mussel. Support units machined from polyoxymethylene (POM) plastic held both sensor and test animal and allowed for fine adjustment of sensor position relative to the magnet (Fig. 2). Each mussel was attached to a 12 mm diameter polycarbonate tube. The end of each tube was profiled with the use of a half round file (20 mm width) to improve its seating on the mussel. The tubes were positioned by attaching each mussel to a horizontal steel plate by its magnet and placing and manipulating the profiled tube onto the uppermost valve until perpendicular to the horizontal plane. Cyanoacrylate glue added to the base of the tube secured it in place. A nylon set screw was used to hold the support tube in position within the frame. A Hall effect sensor (Farnell 179-1388) was waterproofed (white tube in Fig. 2) and fixed within the adjustable frame. The sensor was calibrated for converting output voltage to distance moved (mm) using empty hinged valves within the size range of *M. edulis* used in the experiments. With the valves of the mussels fully closed, the sensor tube was lowered through its support bar until it covered the face of the magnet. The support unit was adjusted to ensure that the sensor face lay in parallel with the magnet face. The sensor tube was then withdrawn vertically until a predetermined voltage (3.15 V) was indicated on the data logger. This voltage served as the baseline from which all valve activity would be measured. A series of shims (0.25–10 mm) were then inserted between the valves at the posterior end of the mussel, with the voltage output generated by each shim width recorded. These measurements were used to generate a calibration curve from which a polynomial function was obtained and used in the subsequent analyses of valve movements. Voltage output from Hall effect sensors was recorded using a National Instruments USB-6009 data logger (Austin, USA), connected to a computer. The data logger was controlled by a simple custom developed LabVIEW programme set to record output voltage at one second intervals from each sensor.

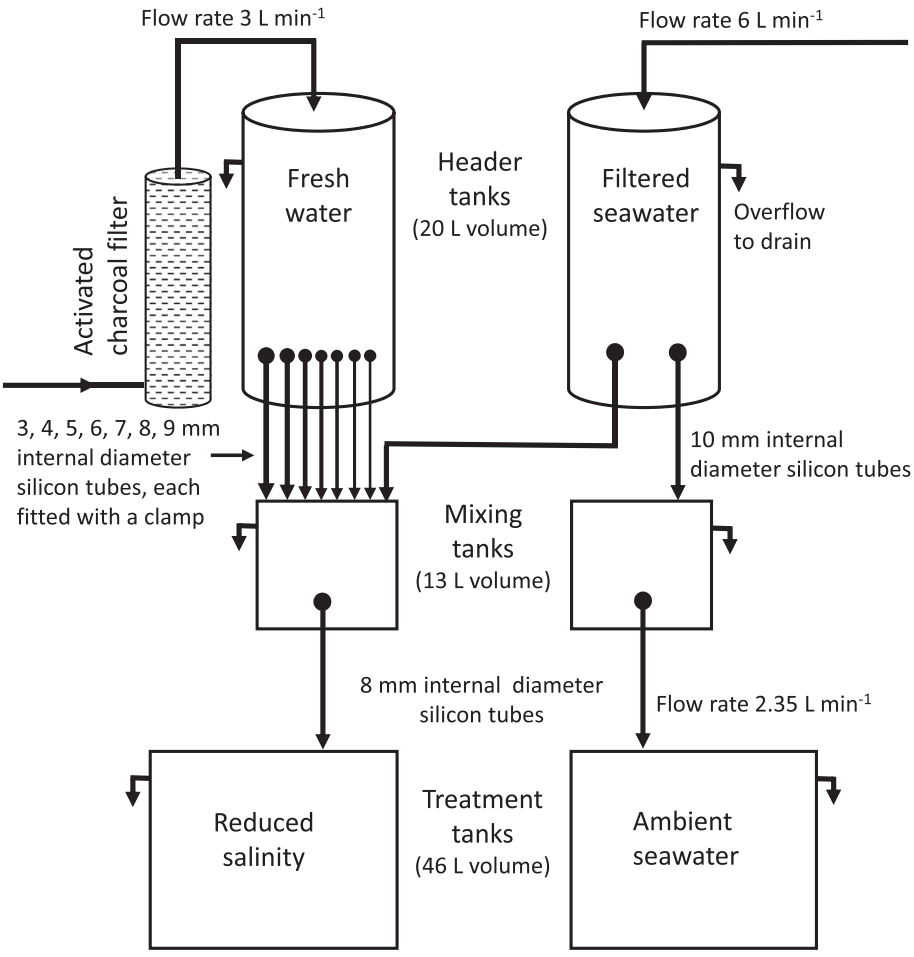
Flow rate 3 L min-1
Flow rate 6 L min-1
Fresh water
Header tanks (20 L volume)
Filtered seawater
Overflow to drain
Activated charcoal filter
3, 4, 5, 6, 7, 8, 9 mm internal diameter silicon tubes, each fitted with a clamp
10 mm internal diameter silicon tubes
Mixing tanks (13 L volume)
8 mm internal diameter silicon tubes
Flow rate 2.35 L min-1
Reduced salinity
Treatment tanks (46 L volume)
Ambient seawater

Fig. 1. Schematic illustration of gravity fed water delivery system.

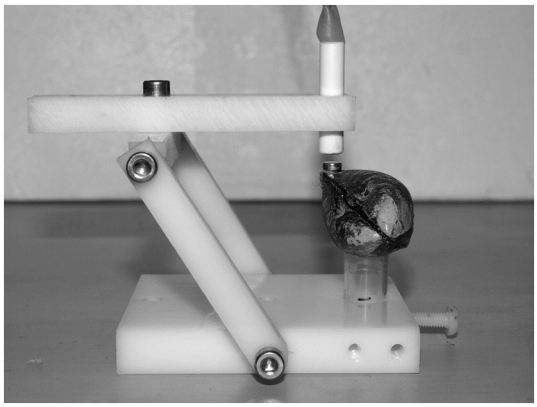

Fig. 2. *Mytilus edulis* with attached magnet lying under a valve gape sensor, mounted within an adjustable support frame.

2.4. Valve gaping behaviour in *M. edulis* during a stepped reduction in seawater salinity

Six days after arrival at the laboratory, 16 *M. edulis* with shell lengths ranging from 59 to 64 mm were taken from the holding tank and prepared as described in Section 2.3. Eight mussels were placed into each of the two treatment tanks (Fig. 1), with both tanks supplied with ambient seawater at salinity 34.3 ± 0.3. Lids were fitted to the tanks and then covered in dense plastic sheeting to further minimize disturbance from changes in light intensity. The area where the tanks were held was constantly lit throughout all experimental procedures. Mussels were left overnight to settle, with recording of valve activity commencing the following morning. Ambient salinity seawater was run through both treatment tanks for the first 24 h of recording. On the second day, a series of freshwater additions were made into the reduced salinity treatment mixer tank, via an array of silicon tubes with a stepwise increase in internal diameter (Section 2.2). Each tube in turn was fully opened for 60 min, then closed as the next tube in line was opened. After the final tube was closed the treatment tank input reverted to full salinity seawater. This procedure generated a gradient of salinity reduction, followed by recovery to full salinity, in the first treatment tank. Recording of valve gape activity continued for a further 5 h after freshwater addition to the mixing tank had ceased. All *M. edulis* were maintained in the treatment tanks in full salinity seawater for the following 14 days. They were fed with algae (Instant Algae, 1800 diet, Reed Mariculture Inc., USA) every 3–4 days using a peristaltic pump to deliver an algal suspension (5 mL of algae concentrate into 0.95 L seawater) at a rate of 3.8 mL min^{-1} into each mixing tank over 2 h. Water temperature in the treatment tanks throughout all experimental procedures was 10.3 ± 0.3 °C.

2.5. Valve gaping behaviour in *M. edulis* held at salinity 25 over 5 days

Further recording of valve activity in *M. edulis* used in Section 2.4 was carried out 14 days after completion of the previous procedure. One group of 8 *M. edulis* was exposed continuously for 5 days to seawater adjusted to salinity 25.0 ± 0.3, with freshwater delivered to the mixing tank through the 7 mm internal diameter feed tube (Section 2.2). Recording continued for a further 2 days after freshwater input ceased, with seawater salinity at 34.3 ± 0.3. Valve activity was recorded in the second group of 8 *M. edulis* held at a salinity of 34.3 ± 0.3 throughout the 7-day experiment. Mussels were not fed

during the exposure to minimize disturbance and to avoid generating valve movements associated with feeding that could mask effects associated with responses to the reduced salinity exposure.

2.6. Haemolymph parameters in M. edulis held continuously at salinity 25 over 5 days

Twenty-four *M. edulis* with shell lengths in the range 56–70 mm, were taken from the holding tank where they had been maintained for the previous 28 days following their delivery to the laboratory, with 12 individuals added to each of the treatment tanks (Fig. 1). Seawater with salinity reduced to 25 ± 0.3 was continuously fed to one tank, with ambient seawater at salinity 34.3 ± 0.3 fed to the other. The mussels were left undisturbed under these conditions for 5 days, after which a haemolymph sample was taken and phagocytosis capacity, total haemocyte count, haemolymph total protein concentration and haemolymph osmolarity was measured in each individual. Approximately 500 μL of haemolymph was taken from the posterior adductor muscle of each individual *M. edulis* using a 1 mL syringe fitted with a 21-gauge needle, after first easing the valves apart along their ventral edge with a bevelled knife. Extracted haemolymph was transferred to a 1.7 mL micro-centrifuge tube (after removing the needle from the syringe) from where aliquots were immediately pipetted into prepared tubes held on ice. 100 μL of haemolymph from each sample was pipetted into the base of a disposable polystyrene culture tube (11 × 75 mm, Sigma Aldrich) preloaded with 100 μL of filtered seawater (0.2 μm) taken from the treatment tank of the mussel being sampled. The mixture was gently flushed with the pipette and the tube returned to stand on ice. For total haemocyte counts, 200 μL of haemolymph was added to a tube containing 200 μL of modified Alsevers' solution (20.8 g glucose, 8 g tri-sodium citrate, 3.36 g EDTA and 22.3 g NaCl into a total volume of 1 L dist. H_2O) containing 1% formalin, with the tube then agitated on a vortexer for 2 s and returned to its rack. For total protein analysis, 10 μL of haemolymph was added to 90 μL of distilled H_2O and mixed on a vortexer for 2 s, then stored at − 20 °C to await analysis. The tube containing the remaining haemolymph from the original sample was returned to sit on ice and provided the 10 μL volume used for measuring haemolymph osmolarity. The time required for sampling and distribution to the pre-prepared tubes averaged 2 min per mussel.

2.6.1. Phagocytosis capacity and efficiency in haemocytes

A BD Accuri C6 flow cytometer (FC) was used to measure phagocytosis capacity and efficiency in *M. edulis* by quantifying uptake of 1 μm fluorescent beads by haemocytes after a fixed period of incubation, following a new experimental protocol. Phagocytosis capacity is defined here as the percentage of haemocytes from each analyzed sample that contain one or more fluorescent beads and phagocytosis efficiency as a measure of enhanced bead uptake, reporting the percentage of haemocytes containing 3 or more fluorescent beads. Sample analysis through the flow cytometer was carried out in series, with approximately 2 min required between samples. The phagocytosis assay protocol has several time dependent components and to ensure that samples were treated as close to an identical manner as possible, steps in the analysis for each sample were staggered at 2 min intervals throughout the procedure (samples were run in batches of 12). Individual polystyrene tubes rather than multi-well plates were used to facilitate effective handling of individual samples through the various steps of the analysis and to allow for more effective detachment of adhered haemocytes from the tube walls immediately prior to analysis through the flow cytometer.

2.6.1.1. Adherence of haemocytes to tube walls. A timer was started immediately following sampling of the first mussel from each batch (Section 2.6). Thereafter further haemolymph samples were prepared at as close to 2 min intervals as possible, until all 12 *M. edulis* in the batch were processed. All prepared tubes were held on ice throughout this procedure. 30 min after the first haemolymph sample was taken all tubes were transferred to a fridge at 8 °C for 30 min. By staggering sample treatment, it was possible to obtain a total period of 60 min incubation for haemocyte adhesion to tube walls for all samples, with 30 min in the fridge and 30 min on ice, with time on ice split between pre and post fridge incubation for most samples. Following the 30 min incubation in the fridge the samples were removed and returned to ice.

2.6.1.2. Incubation of haemocytes with fluorescent beads. All tubes were carefully rinsed twice to remove non-attached haemocytes by using a pipette to deliver and remove 250 μL of 0.2 μm filtered seawater taken from the relevant treatment tank. A 250 μL solution containing yellow-green fluorescent 1 μm carboxylate beads (Life Technologies, 505/515), prepared by adding 25 μL of bead concentrate to 5 mL of filtered treatment seawater, was added to the base of each tube. The wash procedure and addition of fluorescent bead solution was carried out at 2 min intervals between samples, with each completed tube returned to stand on ice. 30 min after the bead solution was added to the first sample tube, all tubes were transferred to the fridge for a further 30 min at 8 °C. Following this step all tubes were returned to ice and washed 3 times with 300 μL filtered seawater taken from the relevant treatment tank to remove beads not associated with haemocytes. Following the 3 washes, a final 300 μL volume of filtered seawater was added to each tube, with the tube then returned to ice. As previously, samples were handled at 2 min intervals.

2.6.1.3. Analysis of haemocytes using the flow cytometer. The final treatment immediately prior to analysis on the flow cytometer was the addition to each tube of 300 μL of modified trypsin-EDTA solution (Sigma T3924) (25 mL dist. H_2O containing 1.65 g NaCl added to 75 mL trypsin-EDTA solution, with 10 mL aliquots stored at − 20 °C) to detach haemocytes from the tube wall. Tubes were incubated at room temperature for a total of 4 min, with 20 s of agitation on a vortexer after 2 min, with this repeated immediately before analysis on the FC. Samples were overlapped during the trypsin incubation so they could be processed through the flow cytometer at 2 min intervals. For each batch of 12 mussels, 2 killed controls were analyzed to account for the incidental presence of fluorescent beads not associated with living haemocytes. These were prepared by replicating the haemocyte adhesion incubation step for two mussels included within the batch and then adding 250 μL filtered seawater with 3% formalin to the tubes for several minutes to kill the haemocytes. These tubes were then washed and the fluorescent bead solution added. The killed controls were then processed and analyzed in the same way as other samples. Following the trypsin incubation all samples were processed on the FC using the following parameters. Fluidic speed 35 μL/min, core size 10 μm, sample volume 50 μL. Software supplied with the FC was used to analyze data and produce plots of fluorescence intensity, together with haemocyte count, size and complexity. The key endpoint for phagocytosis analyses relates to the number of detected fluorescent beads associated with individual haemocytes within each sample. Plots showing haemocyte counts versus fluorescence intensity, generated by the FC software for each sample haemocyte population, were used to derive phagocytosis capacity and efficiency percentages.

2.6.2. Total haemocyte count measurement

For each mussel, 300 μL from a 400 μL solution containing equal volumes of haemolymph and modified Alsevers' solution with 1% formalin (Section 2.6), was added to 20 mL of filtered seawater. 1 mL of this solution was analyzed using a Coulter counter (Multisizer 3) with total haemocyte count recorded for all samples.

2.6.3. Total haemolymph protein measurement

200 μL of prepared Bradford reagent (Sigma B6916) was added to triplicate samples of 10 μL diluted haemolymph (Section 2.6) together with appropriate controls within a microplate and incubated at room

temperature for 30 min. Absorbance was measured at 595 nm using a Thermo Scientific Multiscan Go plate-reader, with results compared against a bovine serum albumin standard curve.

2.6.4. Haemolymph osmolarity measurement

Osmolarity of haemolymph samples and seawater treatments was measured in 10 µL volumes using a Wescor Vapro SS20 Osmometer.

2.7. Data analysis

Mean valve gape averaged over each minute and hour of recording, together with total distance moved between valves 24 h^{-1} were calculated from valve gape distance data sets with a 5 s interval between each measurement. *t*-Tests were used where appropriate to determine if significant differences existed between valve gape data sets. Data from all haemolymph parameters measured are presented in boxplots, where the median and lower and upper quartiles are depicted as boxes, with whiskers representing the maximum and minimum distribution within a 1.5 interquartile range. The Wilcoxon rank-sum test (Mann-Whitney *U* test) was used for statistical testing of all haemolymph parameter data with rejection of null hypothesis set at the 5% probability level ($P < 0.05$). Computation of phagocytosis capacity and efficiency was performed using analytical software supplied with the flow cytometer. All other analyses and plot generation were performed using Microsoft Excel or R.

3. Results

3.1. Valve gaping behaviour in M. edulis during a stepped reduction in seawater salinity

Exposure to a reducing salinity gradient saw an immediate response, with a progressive gradual narrowing of mean valve gape distance until salinity fell below 25 (Fig. 3). At this point an accelerated closure of valves occurred (indicated by dashed lines) and the resultant narrow gape was maintained throughout the remaining salinity reduction. Valves reopened soon after freshwater input to the treatment tanks ceased and mean valve gape distance continued to increase over the following hours until after approximately 3 h, valve gape exceeded that recorded prior to the start of addition of freshwater. *M. edulis* held at salinity 34.3 as controls, showed periodic modest valve gape distance narrowing and reopening behaviour, with mean valve gape remaining relatively high throughout (Fig. 4).

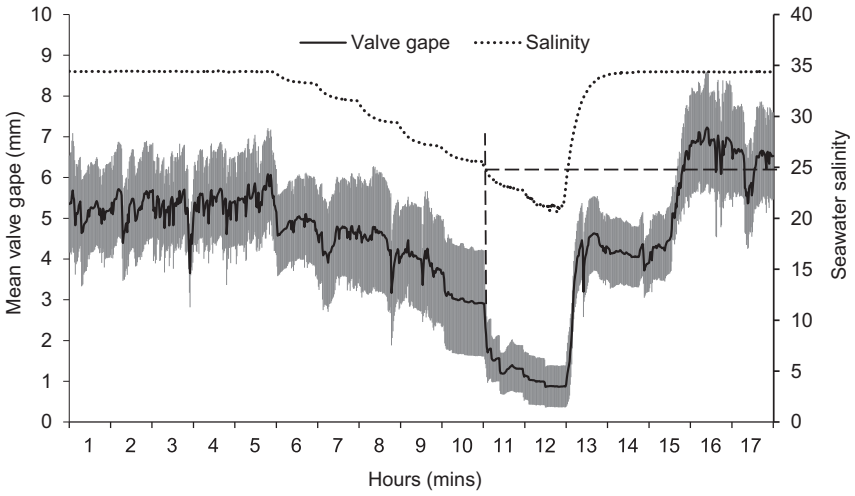

Fig. 3. Mean valve gape measured over 1 min intervals in *M. edulis* exposed to transient changes in salinity. Vertical bars represent ± standard error of the mean (SEM) (n = 8).

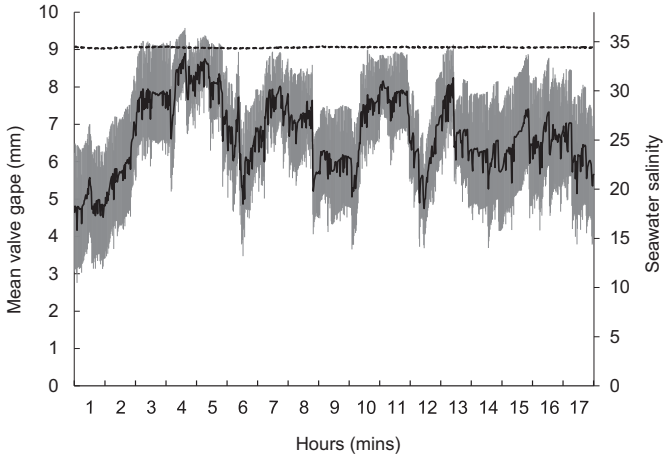

Fig. 4. Mean valve gape measured over 1 min intervals in *M. edulis* held at salinity 34.3. Vertical bars represent ± SEM (n = 8).

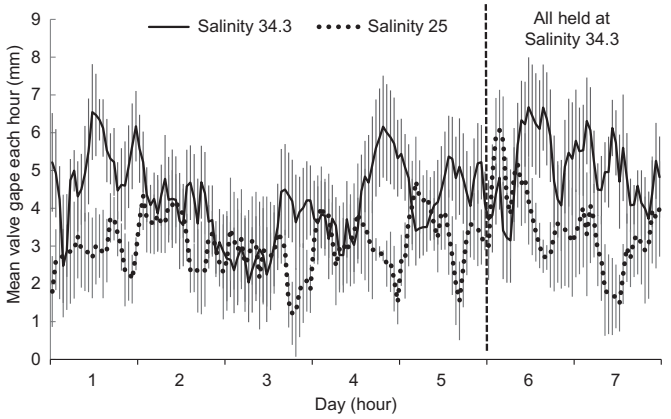

Fig. 5. Mean valve gape measured over 1 h intervals in *M. edulis*. Vertical bars represent ± SEM (n = 7 control, 8 reduced salinity).

3.2. Valve gaping behaviour in M. edulis held at salinity 25 over 5 days

Mean valve gape distance and activity, measured as the mean sum of distance travelled between valves, were lower in *M. edulis* held in reduced salinity seawater than that observed in the controls (Figs. 5 and 6 respectively). The valves of the exposed mussels did however remain largely open and active throughout the 5-day trial, even though the seawater salinity was close to the acute valve closure trigger level

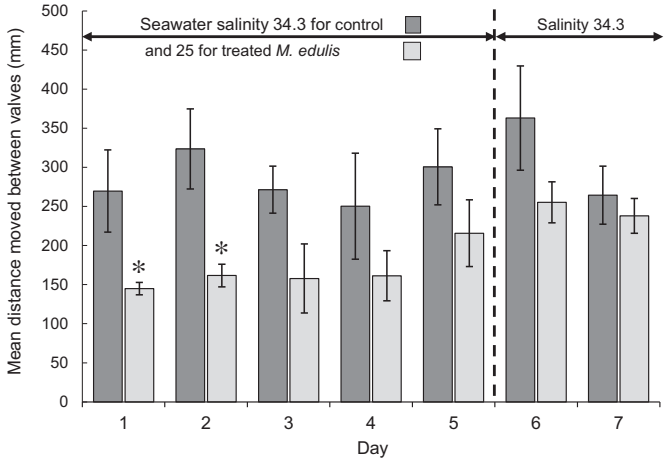

Fig. 6. Mean distance moved by valves within 24 h intervals in *M. edulis* ± SEM (n = 7 control, 8 reduced salinity). * denotes significant difference between exposed and control groups within 24 h periods (two sample *t*-test $P < 0.05$).

established in the previous experiment. There were 3 near-closure episodes during the 5 days of exposure to salinity 25 (Fig. 5), though these were short lived. When full salinity conditions were restored, mean valve gape immediately increased to levels similar to the control mussels for several hours, but then returned to values more typically observed during the reduced salinity period. Valve activity, measured as total distance moved between valves over each 24 h period, was lower in reduced salinity mussels compared to controls, with statistical analyses showing a significant difference between the groups of mussels on day 1 and day 2 (*t*-test, $P < 0.05$). Mean valve activity within the reduced salinity group was however very similar for the first 4 d of the exposure, with the only notable difference being the lower within treatment variability during the first 2 d. The exposed mussels showed an increase in mean activity on day 5 of the exposure and this continued following their return to full salinity seawater conditions (Fig. 6). Inconsistent data from one of the *M. edulis* used in the control group due to a technical failure was omitted from the analyses.

3.3. Haemolymph parameters in M. edulis held continuously at salinity 25 over 5 days

3.3.1. Phagocytosis capacity and efficiency

Phagocytosis capacity was determined by obtaining the percentage of haemocytes within each *M. edulis* sample analyzed associated with one or more fluorescent beads. The results showed that differences in median phagocytosis capacity in the reduced salinity *M. edulis* (49.1%) and controls (42.5%) fell short of statistical significance (Mann-Whitney $U = 39$, $n_1 = n_2 = 11$, $P = 0.173$). Phagocytosis efficiency (percentage of haemocytes associated with 3 or more fluorescent beads) was found to be significantly higher in *M. edulis* exposed to reduced salinity, with a median of 21.1% haemocytes reaching the 3 bead criteria, compared with 12.87% in the control samples (Mann-Whitney $U = 23$, $n_1 = n_2 = 11$, $P = 0.0128$). Boxplots showing the distribution of sample values from each group of *M. edulis* are provided in Fig. 7A and B. Killed control haemolymph samples showed a mean of 4.4% haemocytes associated with 1 or more beads and 0.31% for 3 or more beads (n = 4).

The percentage of haemocytes associated with fluorescent beads generated by the flow cytometer software is calculated from the total number of haemocytes processed through the flow cytometer in each sample volume. This figure is dependent on the total haemocyte count of the original samples (Section 2.6.2) combined with the proportion of these haemocytes successfully adhering to the walls of the sample tube. The percentage of haemocytes from the original sample volumes adhering to tubes was not statistically significantly different between the

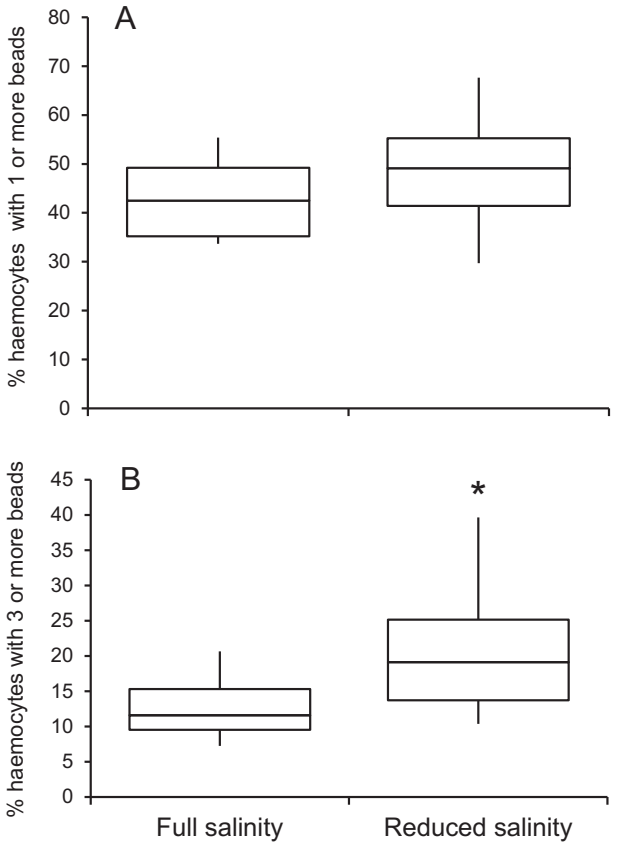

Fig. 7. Boxplots of (A) phagocytosis capacity and (B) phagocytosis efficiency in *M. edulis* (n = 11). * denotes statistically significant difference between groups (Mann-Whitney $U = 23$, $n_1 = n_2 = 11$, $P = 0.0128$).

exposed and control groups (Mann-Whitney $U = 86$, $n_1 = n_2 = 11$, $P = 0.101$). However, the possibility remains that treatment conditions could have the potential to enhance or inhibit the adhesion success of particular haemocyte classes, which in turn could influence apparent phagocytosis performance levels.

3.3.2. Total haemocyte count and protein concentration in haemolymph samples

No significant difference was found in total haemocyte count or total haemolymph protein concentration between control *M. edulis* and those continuously exposed to seawater at a salinity of 25 for 5 days. Boxplots shown in Fig. 8 detail the median values and distribution of (A) total haemocyte counts and (B) total haemolymph protein concentration from both groups.

3.3.3. Haemolymph osmolarity measurement

Haemolymph osmolarity closely matched that of the seawater in the treatment tanks for both groups of *M. edulis*, demonstrating the osmoconforming nature of this species. For *M. edulis* held in full seawater (salinity 34.3), osmolarity was measured at 1016 (± 3) mOsm/kg and for those held at salinity 25, 735 (± 3) mOsm/kg.

4. Discussion

A progressive reduction in salinity established the valve closure trigger threshold salinity to be approximately 24.5 for the test population of mussels used (Fig. 3). It was notable that the inter-individual variability in valve responses for *M. edulis* exposed to these conditions was relatively low, perhaps highlighting a conserved physiological sensitivity based on adaptation to the typical natural salinity fluctuation boundaries found in their home environment. Narrowing of valve

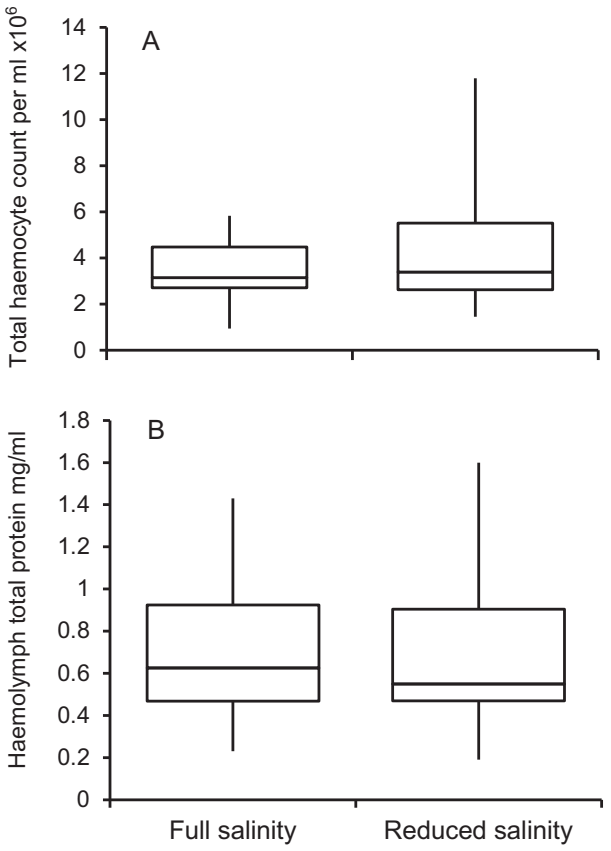

Fig. 8. (A) Total haemocyte count and (B) haemolymph protein concentration in *M. edulis* (n = 11).

gape progressed as salinity was lowered, possibly representing a reduction in pumping rate of seawater across the gills. The relationship between valve gape and pumping rate has been reported in mussels during feeding experiments (Newell et al., 2001; Riisgard et al., 2003) and following long term acclimation of Baltic Sea mussels to a range of salinities (Riisgard et al., 2013). However, a full understanding of the nature of the relationship between acute salinity fluctuation and pumping rate will require further investigation. Re-opening of valves commenced immediately following the cessation of freshwater input, with mean valve gape distance returning to pre-exposure levels and beyond within a few hours. Such a rapid response to rising salinity indicates an effective sensory system, and furthermore supports the presence of a strong physiological imperative to open valves and recommence gill irrigation as soon as possible following even short periods of closure. It is important to note that the salinity threshold triggering a valve closure response varies among populations of *M. edulis* and is dependent on their natural environment and adaptive evolutionary development. Thus coastal *M. edulis* typically exposed to seawater salinity around 34, will close their valves at salinities higher than those populations adapted to live in more permanent low salinity marine areas, such as the White Sea, where mussels have adapted to seawater salinity of around 25 (Sukhotin et al., 2003). A salinity gradient exists within the Baltic Sea, and here *M. edulis* populations can tolerate seawater salinities of 10 or below (Westerbom et al., 2002; Hoeher et al., 2012; Riisgard et al., 2013), although adaptation to this low level of salinity has been reported to lead to dwarfism (Riisgard et al., 2014).

Continuous monitoring of *M. edulis* valve movements during a 5-day exposure to seawater with salinity held just above the acute closure threshold revealed that valves remained largely open and active throughout, albeit at reduced levels when compared against activity in mussels held at full ambient salinity (Figs. 5 and 6). Movement between valves creates changes in pressure in the pallial cavity that assists seawater passage through the valves (Robson et al., 2009; Bamber and Westerlund, 2016), supporting the basal flow generated by the movement of cirri present on the gills (Riisgard et al., 2015). Very low or absent levels of activity between partially open valves could indicate siphon closure, which has previously been reported to precede tight closure of valves in falling salinity conditions in *M. edulis* (Davenport, 1979), though there was no clear evidence of this occurring during the present exposure. There was a significant reduction in valve movements on the first two days in those mussels held in low salinity when compared against controls, though regular activity between valves continued throughout the exposure. The level of valve activity increased in the low salinity group during day 5 of the exposure and this was maintained over the following 2 d after the restoration of full salinity seawater flow. The key finding from this exposure was that tolerance to reduced salinity, close to the level that triggered acute valve closure in the population of *M. edulis* tested, was sustained throughout the full 5 days of the exposure, with no extended periods of valve closure observed. This indicates that the physiological capacity necessary to tolerate reduced salinity at this level did not diminish sufficiently within the exposure timeframe to trigger the isolation response. These observations suggest *M. edulis* are capable of maintaining respiration and feeding activity during sustained reductions in salinity that could follow intense storm events, provided the reduction in salinity does not reach the valve closure threshold.

The absence of extended valve closure periods in *M. edulis* during the 5-day exposure to reduced salinity ensured that the effects of this treatment on haemocyte phagocytosis and the other selected haemolymph parameters were measured in isolation rather than in combination with possible effects due to anoxia within the mussels, following full valve closure. Analysis using flow cytometry revealed that phagocytosis performance in *M. edulis* haemocytes was not negatively affected when mussels were exposed to a drop in salinity of 9 over 5 days. Uptake of fluorescent beads by haemocytes was analyzed following a fixed period of in-vitro incubation, with the percentage of sampled haemocytes containing one or more fluorescent beads, found to be similar in both groups. However, the percentage of haemocytes containing 3 or more fluorescent beads, considered a measure of phagocytosis efficiency (Duchemin et al., 2008; Xue et al., 2001), was significantly higher in reduced salinity mussel samples, when compared against controls held in full salinity seawater (Fig. 7). The reason for this increase, under the given exposure conditions, was unclear and will require further investigation to determine the significance of the response. Measurement of total haemocyte count and haemolymph total protein showed no significant differences between the two groups (Fig. 8). Haemolymph osmolarity was found to be in close equilibrium with that of the seawater treatments for both groups, confirming *M. edulis* as an osmoconformer (Davenport, 1985; Gosling, 2003).

Previous studies have investigated the influence of salinity reduction on immune and haemolymph parameters in bivalves. Bussell et al. (2008) exposed *M. edulis* to seawater with a salinity of 16 over 48 h and reported a significant reduction in both phagocytosis activity and total haemocyte count. It is likely that such a large reduction in salinity from a population of mussels adapted to a salinity of approximately 32 would have triggered a valve closure response and the subsequent development of anoxic conditions within these mussels (Davenport, 1979). In a study that exposed the marine clam *Macrocallista nimbosa* to a range of salinities, significant reductions in phagocytosis were recorded only in those animals held at the lowest salinities of 18 and 21 (transferred from an ambient salinity of 30), where extensive periods of valve closure were also observed (Jauzein et al., 2013). It is likely that anoxic conditions following valve closure are responsible in large part for the detrimental impact on haemolymph parameters recorded in these investigations. Other studies examining the effects of anoxia on haemolymph parameters in marine bivalves have clearly shown that after relatively short periods of valve closure, or exposure to deoxygenated

seawater, both immune function and haemocyte counts were negatively affected (Chen et al., 2007; Matozzo et al., 2005; Pampanin et al., 2002; Yu et al., 2010).

In conclusion, the main objective of the present study was to establish if tolerance to a sustained considerable reduction in seawater salinity would affect phagocytosis performance and total haemocyte counts in *M. edulis*. Results showed that phagocytosis efficiency was actually enhanced after a 5-day exposure to seawater with salinity close to the tolerance limit of the population of mussels tested and that total haemocyte count was not significantly changed. Evidence from previous studies using *M. edulis* and other marine bivalves has shown that even short periods of valve closure can lead to significant negative impacts on phagocytosis and circulating haemocyte numbers, and in some cases mortality. In the context of climate change driven increases in precipitation, the findings point towards sustained phagocytosis efficiency in *M. edulis* during extended periods of reduced salinity that fall within their window of tolerance, followed by a rapid deterioration due to anoxic conditions within the mussel once the tolerance limit is exceeded and the valves are closed. The longevity of any critical reduced salinity event will presumably dictate the severity of the subsequent impact on exposed mussel populations.

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